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Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor-phase extraction and gas chromatography-chemical ionization-mass spectrometry☆

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Abstract

Jasmonic acid and salicylic acid represent important signaling compounds in plant defensive responses against other organisms. Here, we present a new method for the easy, sensitive, and reproducible quantification of both compounds by vapor-phase extraction and gas chromatography-positive ion chemical ionization-mass spectrometry. The method is based on a one-step extraction, phase partitioning, methylation with HCl/methanol, and collection of methylated and, thus, volatilized compounds on Super Q filters, thereby omitting further purification steps. Eluted samples are analyzed and quantified by GC/MS with chemical ionization. Standard curves were linear over a range of 5–1000 ng for jasmonic acid and salicylic acid. The correlation coefficients were greater than 0.999 and the recovery rates estimated between 70 and 90% for salicylic acid and 90 and 100% for jasmonic acid. The limit of detection was about 500 fg by using single ion detection mode. Both, *cis*- and *trans*-isomers for jasmonic acid can be detected. A comparison with established methods indicates the new method to be highly efficient, allowing reliable quantification of both compounds from small amounts of plant material (5–400 mg fresh weight).

Keywords: Jasmonic acid; Salicylic acid; Gas chromatography; Maize; Peanut; Tobacco; Plant defense

Plants respond to insect feeding, pathogen infection, and other environmental stresses with the up-regulation of specific defense mechanisms. Jasmonic acid (JA)[1] and salicylic acid (SA) are thought to be central components of signaling pathways leading to the activation and fine tuning of these defense responses [1,2]. JA, a cyclic derivative of linolenic acid, and MeJA, its methyl ester, have been implicated as playing key roles in inducing herbivore-specific defense responses, and appear to be also involved in some pathogen-induced defense responses [1]. SA and its methyl ester are important signals in the so-called systemic acquired resistance that occurs in plants following pathogen and viral infections [2]. JA and SA are further involved in multiple aspects of plant physiology like development [1], thermogenesis [3,4], and mechanoperception [5,6]. Special interest arose from the findings that both compounds seem to inhibit each other at various steps in their respective biosynthesis and signaling cascades [7,8]. Three different types of methods have been described to estimate both compounds in plants.

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[1] *Abbreviations used:* JA, jasmonic acid; SA, salicylic acid; MeJA, jasmonic acid, methyl ester; MeSA, salicylic acid, methyl ester; dh-JA, dihydro jasmonic acid; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; GC/MS, gas chromatography/mass spectroscopy; GC, gas chromatography; BAW, beet armyworm (*Spodoptera exigua*); SPE, solid-phase extraction; FW, fresh weight; cfu, colony-forming units; PAR, photosynthetic active light.

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Quantification by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) based on specific antibodies has been used successfully [9], but suffered from cross-reactivity with structurally related compounds. High-performance liquid chromatography (HPLC), equipped with a fluorescence detector, has also been used successfully for the quantification of both JA [10] and SA [11], but a complex purification procedure had to be used to separate compounds of interest from an enormous amount of other fluorescent compounds derived from the respective plant sample. Finally, GC/MS technology offers a reliable way to separate, identify, and quantitate compounds and has been applied for the quantification of both JA [12] and SA [13] after derivatization of the carboxylic acids. Electron impact ionization gives structural confirmation but the detection limit and quantification can be negatively affected by components with similar GC retention times as the component of interest. Chemical ionization, on the other hand, gives very little fragmentation and therefore provides very selective detection and high sensitivity [12] but no structural confirmation. Although the final quantification step by GC/MS exhibits a high sensitivity for these compounds, all methods published to date rely on elaborate purification and concentration steps. Typically, ion-exchange columns or hydrophobic interaction columns are used to partially purify and clean the sample material [12–14]. These time-consuming steps severely limit the number of samples that can be processed in a day. A further disadvantage of extensive sample preparation is the loss of compounds during the multiple-step procedure contributing to a reduction of recovery and sensitivity, and, thus, the demand for relatively large sample sizes, typically 1 g of plant material per sample.

Here, we present a new method based on the collection of derivatized and volatilized compounds on polymeric adsorbent (Super Q), serving as the only purification step, and subsequent quantification on GC/MS with positive ion chemical ionization. The method presented here, which speeds up sample processing time in comparison with existing ones, is highly sensitive and allows the quantification of both signaling compounds from milligram amounts of plant material.

Materials and methods

Chemicals

(±) Jasmonic acid, salicylic acid, and methyl salicylate were purchased from Sigma Chemical (St. Louis, MO). Methyl jasmonate and hydrochloric acid (37%) were purchased from Aldrich Chemical (Milwaukee, WI). The polymeric adsorbent Super Q (80/100 mesh) was purchased from Alltech (Deerfield, IL). Dihydro jasmonic acid was purchased from Bedoukian Research (Danbury, CT). Methanol (DriSolv) for methanolysis was purchased from EM Science (Gibbstown, NJ). $^2H_6$-Salicylic acid (98.7 at.% D) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada).

Plant growth

Seeds of corn (*Zea mays* L. cv. Delprim) (Delley Seeds and Plants, Delly, Switzerland) were germinated in vermiculite for 6 days and then transferred to hydroponic containers. The hydroponic solution consisted of 1 mM KNO_3, 0.75 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM NH_4NO_3, 0.5 mM KH_2PO_4, 0.25 mM NaCl, 0.25 mM K_2SO_4, 60 μM Fe–Na EDTA, 50 μM H_3BO_3, 15 μM $MnCl_2 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μ M $CuSO_4 \cdot 5H_2O$, and 0.2 μM $Na_2MoO_4 \cdot 2H_2O$. All plants were maintained in a 12-h photoperiod with 350 μ mol s^{-1} of PAR, 70% relative humidity, and a temperature cycle of 21/26 °C night/day. Plants were used for experiments after 3–4 days in hydroponic solution.

Georgia Green peanut (*Arachis hypogaea* L.) seeds were provided by Drs. Tim Brenneman and Glen Raines (Coastal Plain Experiment Station, University of Georgia, Tifton, GA). Seeds were sown in pairs in 3.78-L pots (16 cm diam) containing a 1/1 (v/v) mixture of commercially available filter sand and Metromix 300 (Scotts-Sierra Horticultural, Marysville, OH). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14-h light:10-h dark light cycle). The greenhouse temperature was kept at 25–30 °C. After emergence, seedlings were thinned to one individual per pot. Each plant received 100 ml of a 3.38 g/liter liquid fertilizer solution [20-20-20 (N-P-K)]; Peters, W.R. Grace (Fogelsville, PA) every 2 weeks starting 1 week after emergence. Five-week-old peanut plants with six fully developed leaves on the main stem and three fully developed leaves on each of two secondary branches were used in all experiments.

Seeds of tobacco (*Nicotiana tabacum* L. strain K326) were sown in a commercial soil mix (MetroMix 300, Scotts-Sierra Horticultural) and kept in a growth chamber at 25 °C. Artificial illumination with metal halide and high-pressure sodium lamps provided a photoperiod of 12:12 h (light:dark) and the relative humidity was kept between 60 and 70%. After 16 days, soil was gently washed off the roots of seedlings with tap water and each seedling was transferred to a 1.0-L plastic cup containing 7 ml nutrient stock solution described by Baldwin and Schmelz [15] with modifications and the final concentrations of each nutrient as follows: 1 mM KNO_3; 0.5 mM NH_4NO_3; 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$; 0.75 mM $MgSO_4 \cdot 7H_2O$; 0.5 mM KH_2PO_4; 0.25 mM NaCl; 0.25 mM K_2SO_4; 0.06 mM Fe–Na-EDTA; 0.05 mM H_3BO_3; 0.015 mM $MnCl_2 \cdot 4H_2O$; 0.002 mM $ZnSO_4 \cdot 7H_2O$; 0.00025 mM $CuSO_4 \cdot 5H_2O$; 0.0002 mM $Na_2MoO_4 \cdot 2H_2O$. The nutrient solution in

each cup was replenished after 10 days. Plants were used for experiments about a month after seeds were planted and had 6–7 leaves including the newly emerged leaf.

Treatment of plants

Alamethicin induction of corn plants

Alamethicin was dissolved in methanol at 1 mg/ml and used at a final concentration of 10 μg/ml in the hydroponic solution. Corn plants were removed from the hydroponic solution and the roots partially cut to fit into a vial containing a 10-ml solution of alamethicin. Controls were also cut and put in 10 ml of pure hydroponic solution containing 10 μl of methanol. Incubation period was 4 h.

Beet armyworm damage on peanut

Beet armyworm (BAW, *Spodoptera exigua* L.) eggs were obtained from the rearing facilities at the USDA-IBPMRL, Tifton, Georgia. Larvae were reared on a pinto-bean artificial diet following the methodology described by King and Leppla [16]. Insects were kept in a biological incubator with a 14-h light:10-h dark cycle at 25 °C. Third-instar larvae were used in all experiments. Peanut plants with 8 fully developed tetrafoliate leaves were treated as follows: (a) healthy (no insect damage), (b) exposed to feeding by 6 BAW for 24 h before tissue was harvested. After removal of the insects from the plants, all the leaves of 6 plants from each of the above treatments were finely ground in liquid N_2 with a mortar and pestle immediately after removal from the plant. All leaf samples were kept in a −70 °C freezer until needed for the analyses.

Pseudomonas syringae infection in tobacco

Prior to plant experiments, *P. syringae* pv. *tomato* DC3000 (Department of Biology, The Pennsylvania State University, University Park, PA) were routinely grown in King's B medium [17] supplemented with 50 μg/ml rifampicin for 18 h at 28 °C on a shaker at 200 rpm. Bacterial cells were collected by centrifugation at 4000*g* for 15 min and resuspended in distilled water. The density of bacterial cell suspensions in water was determined as colony-forming units/ml (cfu/ml) at 600 nm with a Spectro 22 spectrophotometer (Labomed, Culver city, CA) (1 $OD_{600\,nm} = 10^9$ cfu/ml). Plants were inoculated by spraying bacterial suspensions (4×10^7 cfu/ml) with 0.04% Silwet L-77 (OSI Specialties, Friendly, WV) on tobacco leaves until the suspension ran off the leaf surfaces. Control plants were sprayed with 0.04% Silwet L-77 in distilled water only.

Quantification of endogenous hormones

Known amounts of plant material (between 50 and 400 mg) were frozen in liquid nitrogen and ground to a fine powder. Extraction was done by adding 4 ml citric acid 50 mM (in H_2O/acetone (30/70 [v/v]) and the internal standards ($^2H_6$-SA and dihydro-JA (dh-JA), 500 ng each, dissolved in the extraction solution) to the plant material and sonication for 15–20 min at RT. Samples were then centrifuged at 4000*g* (5 min, RT) and the acetone of the supernatant evaporated under a constant air stream. The remaining citric acid phase was extracted with diethyl ether (2×2 ml ether per 4 ml extraction solution) by vortexing and phase separation. The combined ether phases were transferred to a 4-ml screw-cap glass vial and dried under constant airstream to total dryness. The remaining citric acid phase was stored for the analysis of conjugated compounds (see below). Methanolysis was performed by adding 30 μl freshly prepared HCl (37%)/methanol (water-free) (1/2 [v/v]) to the reaction vial, closed with an open-top screw cap, fitted with a Teflon-lined silicone septum, and incubated for 45 min at 70 °C and for an additional 15 min at RT to cool down. To neutralize the highly acidic contents of the vial and to avoid loss of volatile compounds 75 μl of citric acid, trisodium salt (1 M) was injected through the rubber septum and the reaction vial was vortexed. A Super Q filter trap, containing approximately 30 mg of the adsorbent, and a Teflon vent tubing were inserted through a cut in the septum (Fig. 1). The filter was attached to a vacuum source and the vial put in a heating block at 80 °C and volatilized compounds were trapped until the sample was totally dry (approximately 3–5 min). The flow rate through the collection filter was 450–500 ml/min. However, no significant differences in the trapping of volatiles could be observed with flow rates ranging from 10 to 1000 ml/min. The compounds were eluted with 150 μl dichloromethane from the adsorbent. To extract the

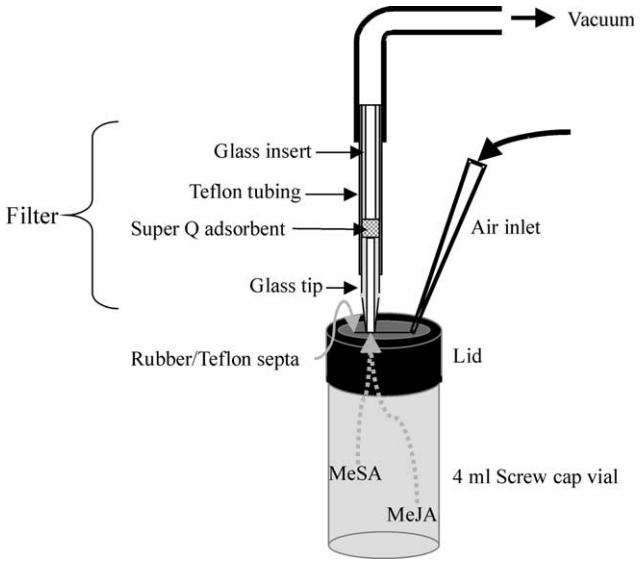

Fig. 1. Trapping of methylated plant signaling compounds on Super Q filters.

conjugated SA, the remaining citric acid phase was hydrolyzed by adding 10 µl concentrated (37%) HCl and heating the capped vial at 95 °C for 1 h. After cooling to RT, a new aliquot of internal standard was added and the samples were reextracted with ether and processed further as described above.

An HP 6890 gas chromatograph equipped with a split/splitless injector (splitless mode, 250 °C, injection volume 1 µl), interfaced to an HP 5973 mass spectrometer (Hewlett-Packard, Palo Alto, CA) operated in chemical ionization mode with isobutane as the ionization gas, was used for the analysis. Compounds were separated on an HP-1MS (30 m × 0.25 mm × 0.25 µm) column held at 40 °C for 1 min after injection, and then temperature programmed at 15 °C/min to 250 °C (10 min), with helium as the carrier gas (constant flow rate 0.7 ml/min). Measurements were carried out either in total ion count (TIC) or in selected ion count (SIC) with the following program: 3.5–10.31 min, ions $(m+1)^+$ 153 (methyl salicylate (MeSA)), 157 (methyl $^2H_4$-salicylate ($^2H_4$-MeSA); 10.31–13.50 min, ions $(m+1)^+$ 225 (methyl jasmonate (MeJA)), 227 (methyl dihydro-jasmonate (dh-MeJA)). Retention times were as follows: MeSA, 8.62 min; $^2H_4$-MeSA, 8.60 min; *trans*-MeJA 12.48 min; *cis*-MeJA 12.71 min; *trans*-dh-MeJA, 12.50 min; *cis*-dh-MeJA 12.75 min.

All compounds were identified by comparison of their retention time with pure commercially available standards. Further identification was performed by selecting samples for GC/MS analysis in electron impact ionization mode and comparison of fragmentation and retention time with pure compounds. Quantification of JA and SA was done by correlating the peak area (extracted ion) of the compound with that of the respective internal standard. Although $^2H_6$-SA was added as an internal standard we actually measured $^2H_4$-SA due to loss of two deuterium ions during methanolysis at the carboxylic acid group and the exchange of the acidic –OD to –OH due to the acidic environment during sample preparation.

Recovery and reliability

The reliability of the method was tested by adding 0, 5, 10, 50, 100, 500, and 1000 ng of SA and JA and 500 ng of the respective internal standard to 50–60 mg of frozen and ground corn leaf material and then processed as described above. Preliminary experiments showed that a plant matrix was necessary for reproducible results. The recovery rate was calculated by comparing the response in GC peak area units of the internal standard in these samples with known amounts of the internal standard methyl esters that had been prepared by treatment with Methanol/HCl (2/1 [v/v]) for 45 min at 70 °C, neutralized as above, and then directly extracted with 200 µl of dichloromethane.

Linear response for small plant samples

Corn plants were induced with alamethicin, which is known to induce both JA and SA [7]. After 4 h of incubation plants were immediately frozen and ground. Small amounts of the plant material (5–50 mg) were taken from induced and control plants to detect and quantify the two signaling compounds. Due to the small sample size the following modifications were performed: first extraction with 2 ml of acetone/citric acid (70/30 [v/v]); phase partitioning with 2 ml of diethyl ether; elution of bound material from Super Q filter material with 150 µl dichloromethane.

Other methods used for comparison

Jasmonic acid (according to Weber et al. [14] with modifications Original at: http://www.unil.ch/ibpv/WWWFarmer/WWWOxylipins/Docs/method.htm)

Homogenized leaf tissue samples of approximately 1 g were extracted in 3.5 ml methanol with 500 ng of the internal standard dh-JA. After 30 min in a sonicating bath, each sample was mixed with 1.5 ml of purified H_2O, and centrifuged at 3000*g* for 5 min. The supernatant was adjusted to pH 8.5 with aqueous 1 M NH_4OH and kept on ice. Solid phase extraction (SPE) cartridges (reverse-phase C18, 12 ml, Mallinckrodt Baker, Griesheim, Germany) were washed with 8 ml each of 100% methanol, followed by 70% methanol. Each sample was passed through the SPE cartridge followed by 7 ml of 75% methanol. All eluate was collected, adjusted to pH 3.5 with 10% [v/v] formic acid, and the volume was raised to a total of 50 ml with H_2O. The SPE cartridges were cleaned and conditioned for reuse with 5 ml each of 0.8% formic acid in methanol, 100% methanol, diethyl ether, 100% methanol, and finally 10 ml H_2O. The samples were then reloaded on the cartridges and washed with 7 ml each of 85:15 H_2O:ethanol [v/v] and H_2O. After all water was removed from the columns, the oxylipin fraction was eluted with 10 ml diethyl ether. The volume of the eluate was reduced under N_2 to less than 2 ml, transferred to a 2-ml reaction vial where it was totally dried under N_2. Methanolysis was performed by incubating with 30 µl of a 1/2 [v/v] HCl:methanol mixture for 12 h at 30 °C. The HCl:methanol was then completely removed under a stream of N_2 gas and each sample was brought up to 75 µl in dichloromethane. Samples were analyzed by GC/MS as described above.

Jasmonic acid and salicylic acid (according to Engelberth et al. [7] with modifications)

The quantification of endogenous JA and SA followed the protocol of McCloud and Baldwin [18] originally developed for determination of endogenous jasmonic acid. Treated leaves (0.3–0.4 g of tissue) were

frozen and ground under liquid nitrogen. The resulting powder was suspended in a solution of acetone and 50 mM citric acid (70/30 [v/v]). As internal standards $^2H_6$-SA and dh-JA (500 ng) were added. Samples were sonicated for 15 min and then centrifuged at 4000*g* (5 min, RT) and the acetone of the supernatant was evaporated under a constant air stream. The remaining citric acid phase was extracted with diethyl ether (2×2 ml ether per 4 ml extraction solution) by vortexing and phase separation. The extracts were then loaded onto an anion-exchange solid-phase extraction cartridge (500 mg of sorbent, aminopropyl, Varian, Darmstadt, Germany). After loading, the cartridges were washed with 7.0 ml of a solvent mixture of trichloromethane:2-propanol (2/1 [v/v]). Bound JA, SA, and the corresponding standards were eluted with 10 ml of diethyl ether:acetic acid (98/2 [v/v]). After evaporation of solvents and esterification of the residue by methanolysis as described herein, volatilized compounds were trapped on SuperQ filter material and eluted with 200 µl dichloromethane.

Salicylic acid (according to O'Donnell et al. [19])

Leaf tissue was harvested, frozen in liquid nitrogen, and stored at −70 °C. Salicylic acid and SA conjugates were extracted and analyzed as described [11]. In brief, 0.5 g tissue was ground in liquid nitrogen and then extracted with 3 ml 90% methanol followed by 2 ml 100% methanol. The combined extracts were then divided into two, dried down, and resuspended in either 2.5 ml 5% [v/v] trichloroacetetic acid (for free SA determination) or phosphate buffer (for total SA determination). Conjugated forms of SA were hydrolyzed by boiling for 30 min in acidified phosphate buffer. Both fractions were then extracted twice with an equal volume of ethylacetate:cyclopentane:isopropanol (100:99:1 [v/v]), dried down, and resuspended in 20% [v/v] methanol. Salicylic acid was identified and quantified by reverse-phase HPLC on a 5-µm C-18 column (Beckmann Ultrasphere, Fullerton, CA; 4.6×250 mm) and detected using a Waters 474 scanning fluorescence detector (excitation energy 295 nm, emission energy 400 nm). Identification and recovery of SA were determined by spiking a noninduced sample with a known amount of an authentic standard.

Results

General remarks

The estimation of the sensitivity of the GC/MS was the first step in the evaluation of the method. Chemical ionization in single ion mode revealed the highest sensitivity for both compounds, JA and SA. By using methylated standards, retention times were estimated and a method was established to collect only relevant ions in a designated time window as shown in Fig. 2. The retention times for the methylated compounds were 8.62 min for $^2H_4$-MeSA (*m/z* 157), 8.64 min for MeSA (*m/z* 153), 12.48 min for *trans*-MeJA (*m/z* 225), 12.71 min for *cis*-MeJA (*m/z* 225), 12.54 min for *trans*-dh-MeJA (*m/z* 227), and 12.75 min for *cis*-dh-MeJA (*m/z* 227).

After establishing the GC program the sensitivity of the GC/MS was evaluated by using dilutions of known amounts of MeJA and MeSA in dichloromethane. A GC/MS detection limit of 500 fg was estimated for both pure compounds. Quantification was always performed on extracted SI chromatograms. Furthermore, MeSA and $^2H_4$-MeSA exhibit almost the same retention time so that these two compounds can only be separated by single ion extraction (Fig. 2B).

During sample preparation unfiltered house air was used to facilitate the evaporation of organic solvents. Although there is a certain probability of contamination we found no difference in the use of either nitrogen gas

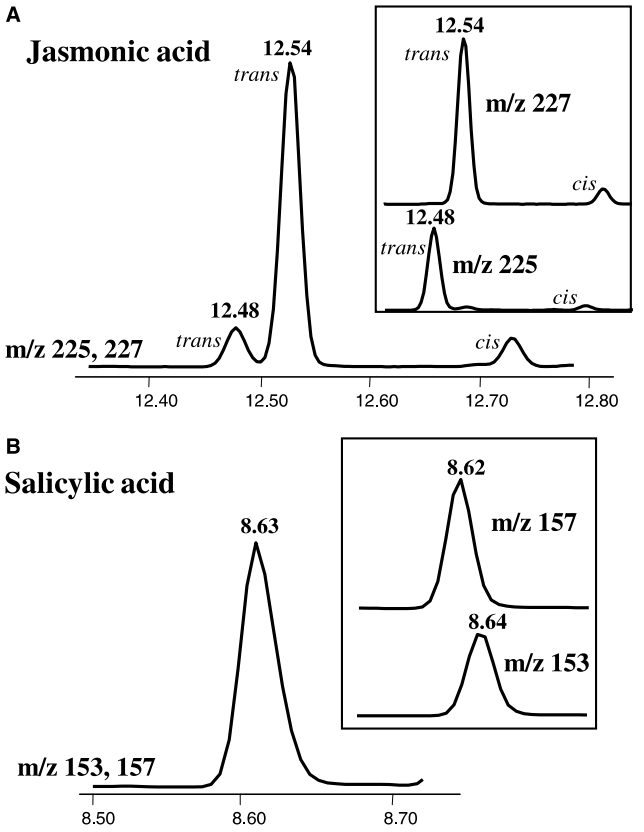

Fig. 2. Ion traces of the relevant $(m + 1)^+$ ions for the methyl esters of jasmonic acid, dihydro jasmonic acid, $^2H_4$-salicylic acid, and salicylic acid. (A) Collected ions in this window were jasmonic acid ($(m + 1)^+$ 225) and dihydro jasmonic acid ($(m + 1)^+$ 227). *Trans*- and *cis*-isomers of JA and dh-JA exhibited a constant ratio of 0.95:0.05. (B) Collected ions in this window were $^2H_4$-salicylic acid ($(m + 1)^+$ 157) and salicylic acid ($(m + 1)^+$ 153). Note that jasmonic acid and its respective internal standard are separated, but salicylic acid and $^2H_4$-salicylic acid have almost identical retention times and can only be separated by single ion extraction.

or house air. Therefore, we preferred house air because it reduces the costs and did not negatively affect the results.

A further critical point is the use of an appropriate internal standard. Dh-JA has been reported to occur in plants. Therefore all plants used were checked previously for this JA derivative. Care must be taken by using deuterated compounds as internal standards. $^2H_6$-SA as internal standard lost two deuterium ions, as described above, resulting in $^2H_4$-SA as the actual internal standard with a relative abundance of 82%, which has to be taken into account when calculating endogenous SA.

The recovery for SA and $^2H_6$-SA (measured as $^2H_4$-SA) was 70–90%, for jasmonic acid and dh-JA, 90–100%. Thus, derivatization and volatilization of JA, dh-JA, SA, and $^2H_6$-SA by methanolysis were nearly quantitative as indicated by the high recovery rate of the procedure.

Accuracy and precision

Standard curves were measured twice with 4 replicates for each added amount of compound. The estimation of the recovery rate revealed that JA and its corresponding internal standard dh-JA as well as SA and $^2H_6$-SA exhibit the same properties expressed as recovery rate. Previous experiments showed that by using small amounts of plant material as a background matrix the recovery for low amounts of added compounds was much higher compared to samples without this. With plant material serving as a matrix the standard curve was linear over a range of 0–1000 ng of added compound for both jasmonic acid and salicylic acid with a correlation coefficient of 0.999 for JA and 1 for SA (Fig. 3).

Standard curves for small plant sample sizes (5–50 mg of plant material) were established by comparing control plants with alamethicin-induced plants. The effect of alamethicin on corn plants was established in a separate experiment with the normal amount of plant material used (data not shown).

SA was strongly induced with alamethicin. This is reflected by the regression curve for the induced plant samples leading to 2.77 μg of salicylic acid per gram plant material (correlation coefficient 0.98). In control plants the level reaches about 35 ng/g FW (correlation coefficient 0.82) (Fig. 4A).

The established regression curve for jasmonic acid in control plants exhibited the highest variation, but led to about 11 ng/g plant material, which is consistent with previous results from corn plants. In alamethicin-induced plants the regression curve exhibited a correlation coefficient higher than 0.9, mainly due to the higher levels of jasmonic acid (54 ng/g FW) in the plant tissue (Fig. 4B).

Comparison with established methods

To further verify the quality of the vapor-phase procedure it was compared with well-established methods. Different plant species and two different types of treatment were used in order to prove the broad applicability of the developed procedure.

Jasmonic acid

Herbivore damage is known to induce JA [1]. Therefore, we used caterpillar infestation on peanut plants to monitor JA. The protocol according to [14] was used due to its common use in the literature and compared with the herein-described vapor-phase extraction (Table 1). Plant material was taken from the same sample, which was prepared as described previously. The commonly used method described by Weber et al. [14] gave levels of 21.8 ng/gFW, whereas the

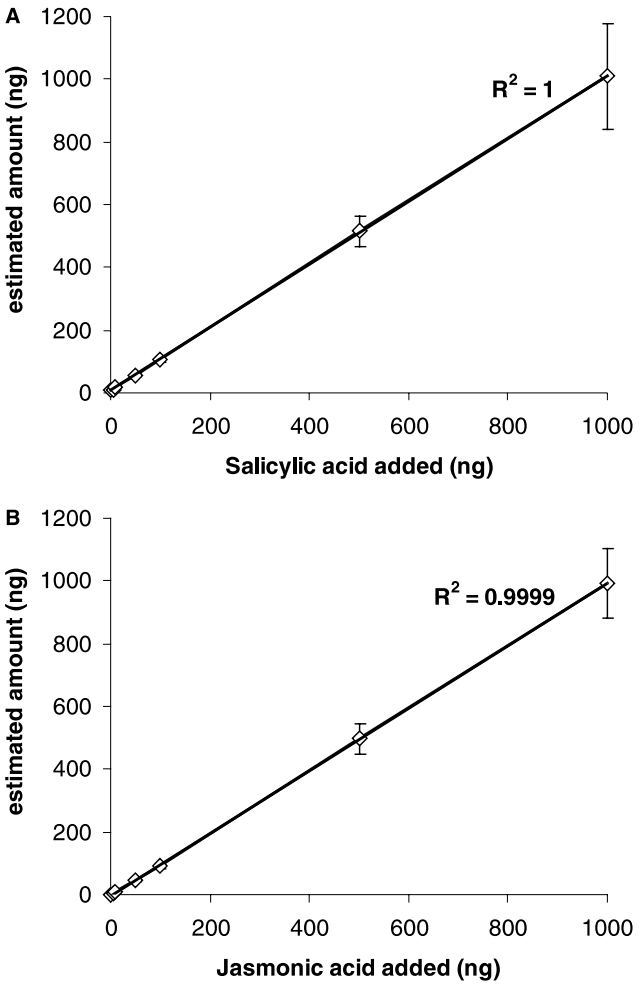

Fig. 3. Accuracy of the quantitative analysis of jasmonic acid and salicylic acid. The amounts of 0, 5, 10, 50, 100, 500, and 1000 ng salicylic acid (A) and jasmonic acid (B) were added to approx 50 mg of plant material of *Z. mays*. Samples were then extracted and analyzed as described under Materials and methods ($n = 8$). R^2, correlation coefficient.

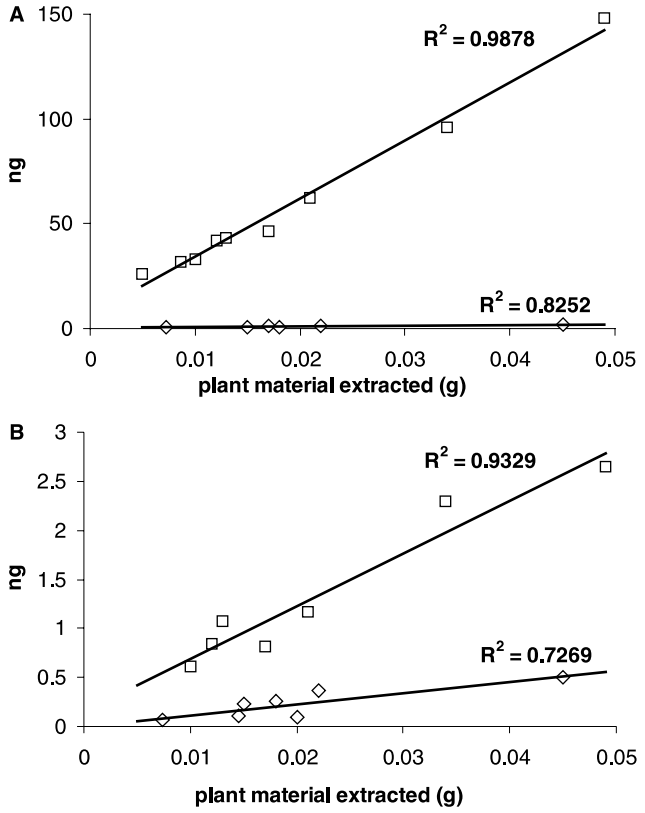

Fig. 4. Precision of the quantitative analysis of salicylic acid (A) and jasmonic acid (B). Small amounts of plant material from alamethicin-induced (□) and uninduced (◇) corn plants (*Z. mays*) were extracted according to the procedure described under Materials and methods. Because of the small sample size, primary extraction was performed with 2 ml of acetone/citric acid and phase separation was done with 2 ml of ether. Quantities are expressed as ng/sample. Sample size was between 0.005 and 0.05 g of plant material. R^2, correlation coefficient.

Table 1
Comparison of different methods for the quantification of jasmonic acid ($n = 4$)

Peanut plus *Spodoptera exigua*		
Method	Jasmonic acid (ng/g FW)	
	Control	Herbivory
Weber et al. [14]	No peaks detectable	21.8 ± 1.9
Vapor-phase extraction	3.23 ± 2.3	31.2 ± 2.0

vapor-phase extraction method gave 31.2 ng/gFW for caterpillar-induced plant material (Table 1). However, using the established method [14] the JA levels were below the detection limits in the control plants.

Salicylic acid

To compare different extraction procedures tobacco plants were inoculated with *P. syringae* pv. *tomato*, which induce hypersensitive response and accumulation of SA in tobacco. Three different methods were used to estimate SA from the same plant material (Table 2) and the results compared with regard to sensitivity and reliability. The two methods based on GC/MS analysis exhibit almost identical results, although the comparison of the recovery rate expressed as relative ion intensity by MS quantification revealed that vapor-phase extraction delivers more ions, resulting in better quantification from small sample sizes. The use of HPLC for quantification revealed a lower amount of compounds compared to the other method.

Discussion

We describe a simple sample preparation method for routine analysis of both jasmonic acid and salicylic acid. Using the sample preparation as described above, 50 samples per day can be routinely analyzed with high precision. Furthermore, the method allows the quantification of these signaling compounds in small amounts of plant material making it suitable for the investigation of local events in plants.

The use of internal standards serves well for the automatic correction of losses during sample preparation as well as for the quantification during mass spectrometric analysis. However, care must be taken in the use of the appropriate internal standards. They should not be abundant in the plant tissue analyzed and, as described for $^2H_6$-SA, the degradation of the labeled standard must be estimated and taken into account when calculating the amount of endogenous compound. The extraction of single ions allowed the separation of $^2H_4$-SA and SA, although no baseline separation was achieved.

Table 2
Comparison of different methods on the amount of extracted compounds ($n = 4$)

Tobacco plus *Pseudomonas syringae* pv. tomato				
Method	Total amount of compound (ng/g FW)		Relative ion intensity	
	SA	Conjugated SA	SA	Conjugated SA
O'Donnell et al. [19] HPLC	207 ± 31	4733 ± 266	—	—
Engelberth et al. [7] GC/MS	814 ± 39	5169 ± 275	4181 ± 1766	17276 ± 3187
Vapor-phase extraction GC/MS	697 ± 27	4979 ± 127	7244 ± 3389	40312 ± 6360

The avoidance of multiple cleaning steps by trapping volatile compounds from crude methylated extracts reduces the loss of compounds as indicated by the high recovery rate for JA and SA. Furthermore, the new method facilitates the extraction of conjugates of salicylic acid from one sample without splitting after the first extraction. This leads to an increase of both free salicylic acid and its conjugate in one sample, enabling the quantification of total SA from small sample sizes. Various authors have demonstrated the successful use of Super Q adsorbent to collect volatile compounds [20,21]. The use of this method avoids multiple sample preparation steps, thereby avoiding loss of material. It is quantitative and handy to use and, although these filters are not commercially available, they are easily made and can be reused many times. Care must be taken in the selection and use of the internal standard.

In order to compare this method with other, well-established methods, we chose those which use either GC/MS technology or, in case of SA, HPLC. The comparison revealed that none of the other methods tested exhibits a significant advantage in the estimation of the compound. In most cases better results were obtained by using the vapor-phase extraction method as indicated by higher recovery rates. The use of a closed system from methanolysis might contribute to a better recovery and might also explain the differences concerning JA in the control plant extracted by the method of [14]. Although there were no significant differences between the extraction with methanol/water and acetone/citric acid/water we preferred the later. This was mainly due to the fact that with methanol/water extraction spontaneous methylation occurred leading to loss of compounds during evaporation of the solvent. Additionally acetone has a lower boiling point (56 °C) than methanol (63.8 °C) facilitating faster evaporation.

To our knowledge there are only two methods described which were capable of quantifying both JA and SA from one plant sample. Engelberth et al. [7] used a comparable method for the simultaneous quantification, which followed the original protocol of McCloud and Baldwin [18], except that it was extended to SA. It has been shown that SA can be extracted in the same manner as JA, which was also demonstrated herein. However, using aminopropyl columns to enrich these two compounds extended the procedure and finally, opening of the reaction vial after methylation might reduce the yield making it inconvenient for small sample sizes. Furthermore, the method required a large amount of solvent for washing and eluting the column. The second method for the quantification of both JA and SA as well as MeJA of Wilbert et al. [22] is based on capillary liquid chromatography coupled to electrospray tandem mass spectrometry. Although the method is convenient for all of these compounds it required a cleaning step by separation on a C18-column. And secondly, the method required specific equipment less commonly available than GC/MS. We were unable to compare this method with ours. It seems that both methods are very simple to use and a decision on which method to use depends on the available equipment. The herein-described procedure may be adapted for quantification of other compounds providing they are stable and an appropriate internal standard is available.

Thus, the method presented here exhibits a new procedure for the reliable quantification of at least two different plant signaling compounds from small amounts of plant material in a time- and resource-efficient manner. Work is in progress to extend this method to other acidic signaling compounds and their respective precursors.

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